Increased Formation of Distinct F_2 Isoprostanes in Hypercholesterolemia

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Background—F_2 isoprostanes are stable, free radical–catalyzed products of arachidonic acid that reflect lipid peroxidation in vivo.

Methods and Results—Specific assays were developed by use of mass spectrometry for the F_2 isoprostanes $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI and arachidonic acid (AA). Urinary excretion of the 2 F_2 isoprostanes was significantly increased in hypercholesterolemic patients, whereas substrate AA in urine did not differ between the groups. $iPF_{2\alpha}$-III (pmol/mmol creatinine) was elevated ($P<0.0005$) in homozygous familial hypercholesterolemic (HFH) patients (85 ± 5.5; n=38) compared with age- and sex-matched normocholesterolemic control subjects (58 ± 4.2; n=38), as were levels of $iPF_{2\alpha}$-VI (281 ± 22 versus 175 ± 13; $P<0.0005$). Serum cholesterol correlated with urinary $iPF_{2\alpha}$-III ($r=0.41$; $P<0.02$) and $iPF_{2\alpha}$-VI ($r=0.39$; $P<0.03$) in HFH patients. Urinary excretion of $iPF_{2\alpha}$-III (81 ± 10 versus 59 ± 4; $P<0.05$) and $iPF_{2\alpha}$-VI (195 ± 18 versus 149 ± 20; $P<0.05$) was also increased in moderately hypercholesterolemic subjects (n=24) compared with their controls. Urinary excretion of $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI was correlated ($r=0.57$; $P<0.0001$; n=106). LDL $iPF_{2\alpha}$-III levels (ng/mg arachidonate) were elevated ($P<0.01$) in HFH patients (0.32 ± 0.08) compared with controls (0.09 ± 0.02). The concentrations of iPF_2-III in LDL and urine were significantly correlated ($r=0.42$; $P<0.05$) in HFH patients.

Conclusions—Asymptomatic patients with moderate and severe hypercholesterolemia have evidence of oxidant stress in vivo. (***Circulation.* 1998;98:2822-2828.**)

Key Words: eicosanoids ■ cholesterol ■ atherosclerosis

Oxidation of LDL is thought to play a critical role in atherogenesis.[1,2] However, evaluation of antioxidants in cardiovascular disease has yielded conflicting results.[3–8] Insight into the role of excessive free radical generation in human disease has been constrained by the limitations of current indexes of oxidant stress in vivo.[9,10]

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Isoprostanes are chemically stable, free radical–catalyzed products of arachidonic acid (AA) that are structural isomers of conventional prostaglandins[11,12] for which a novel nomenclature[13] has recently been proposed (Figure 1). We have recently immunolocalized 1 of these compounds, $iPF_{2\alpha}$-III (formerly known as 8-*iso*-$PGF_{2\alpha}$), to monocyte/macrophages and vascular smooth muscle cells in human atherosclerotic plaque.[14] Furthermore, we and others[15–17] have demonstrated that this compound is formed in LDL when it is oxidized in vitro. Given that isoeicosanoids are formed initially in situ in membrane phospholipid and are then susceptible to phospholipase-dependent cleavage, circulation, and excretion in urine,[18] we wished to investigate their formation as indexes of oxidant stress in human hypercholesterolemia and atherosclerosis.

Methods

Clinical Studies

Volunteers and patients were enrolled at the hospitals of the University of Pennsylvania, the Children's Hospital of Philadelphia, the University of Naples, and the University of Milan.

Three studies were performed. In the first, urinary isoprostane excretion was measured in 38 subjects (24 males and 14 females aged 3 to 43 years;) with homozygous familial hypercholesterolemia (HFH), defined as patients with childhood cutaneous or tendonous xanthomata, fasting total cholesterol levels of >500 mg/dL, and normal triglyceride levels (<200 mg/dL) (Table 1). These patients

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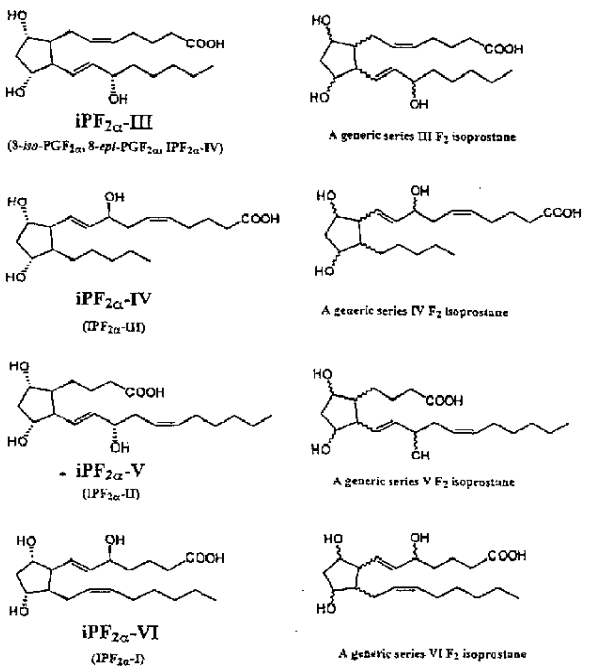

Figure 1. F_2 Isoprostane structures. Bold type denotes proposed nomenclature. Names in parentheses represent previously used terminology.

were enrolled prospectively in a consecutive manner as they were admitted to the clinical research centers of the respective hospitals over a 1-year period. None of the subjects were cigarette smokers. Six patients gave a history of antioxidant consumption. None were diabetic or hypertensive, and most were white (2 blacks and 1 Asian). Patients who were receiving plasmapheresis had not undergone this treatment for ≥1 week before assessment. All patients underwent a history and physical examination and were admitted to the clinical research center for a 48-hour period. Overnight 12-hour urine samples were collected before fasting blood samples were taken for the assessment of lipoproteins, and coronary angiography

TABLE 1. Characteristics of Patients With HFH and Age- and Sex-Matched Controls

Characteristic	HFH (n=38)	Control (n=38)	P
Age, y	21±2.3	20±1.9	NS
Sex, male/female	24/14	24/14	NS
Coronary disease	22/38	0/38	<0.0001
Medications			
Aspirin	27/38	0/38	<0.0001
Statins	26/38	0/38	<0.0001
Cholestyramine	8/38	0/38	0.003
Niacin	5/38	0/38	0.02
Plasmapheresis	10/38	0/38	0.0007
Cholesterol, mg/dL	548±30	180±8.0	<0.0001
LDL, mg/dL	480±30	99±3.0	<0.0001
HDL, mg/dL	35±2.1	49±1.6	0.0002
Triglycerides, mg/dL	138±17	90±6.2	NS

Values for continuous variables are expressed as mean±SEM.

TABLE 2. Characteristics of Patients With HC and Age- and Sex-Matched Controls

Characteristic	HC (n=24)	Control (n=24)	P
Age, y	41±2.9	37±2.2	NS
Sex, male/female	16/8	16/8	NS
Coronary disease	3/24	0/24	NS
Medications			
Aspirin	3/24	0/24	NS
Statins	2/24	0/24	NS
Cholesterol, mg/dL	290±9.0	171±6.0	<0.0001
LDL, mg/dL	206±10	102±4.3	<0.0001
HDL, mg/dL	47±2.6	52±2.4	NS
Triglycerides, mg/dL	173±19	83±6.3	0.05

Values for continuous variables are expressed as mean±SEM.

was performed. An age-, sex-, smoking-, and center-matched sample of subjects (aged 3 to 54 years; 24 males and 14 females) was used as a control group (n=38). They underwent similar collections and fasting lipoprotein assessment. Urinary arachidonate was measured in a subset of HFH patients (aged 4 to 43 years; 6 males and 6 females) and matched control subjects (aged 5 to 43 years; 6 males and 6 females).

In a second study, we measured $iPF_{2\alpha}$-III corrected for LDL protein in unstimulated LDL from a sample of HFH subjects (aged 5 to 54 years; 10 males and 6 females) and matched control subjects (aged 11 to 54 years; 10 males and 6 females). Blood samples were drawn for LDL isolation on the same day as the urine collection and lipoprotein determination described above. Given the possible differences in substrate concentrations between the groups, we also corrected LDL $iPF_{2\alpha}$-III values for cholesterol and for AA in a subgroup of these HFH subjects (aged 20 to 54 years; 5 men and 2 women) and control subjects (aged 28 to 54 years; 5 men and 2 women).

The third study involved recruitment of a group of adults presenting consecutively at lipid clinics over a 2-week period with moderate hypercholesterolemia (HC; patients with fasting total cholesterol levels >240 mg/dL; n=24) and an age-, sex-, and smoking-matched group of normocholesterolemic controls (n=24) (Table 2). All patients were on lipid-lowering diets. There were 4 smokers each in the HC and control groups. No patient gave a history of antioxidant consumption. None of the patients were hypertensive or diabetic, and all were white. Twelve-hour urine samples were collected for the determination of urinary F_2 isoprostanes, and fasting blood samples were drawn during this period for lipoprotein assessment.

Isoprostane and AA Analysis

Urinary isoprostanes were measured as previously described.[19,20] Urinary levels of AA were also measured by gas chromatography/mass spectrometry. Briefly, after addition of [$^2H_8$]-AA to urine, the pH was lowered to <3.0, and the sample was extracted twice before derivatization with pentafluorobenzyl bromide. The sample was then subjected to thin-layer chromatography and analyzed by gas chromatography/mass spectrometry, monitoring ions at m/z 303 and 311 for 50 ms each. The retention time of AA is ≈6 minutes. Total AA levels (after hydrolysis with 15% aqueous KOH solution) in LDL were assayed by use of a similar approach.

LDL for $iPF_{2\alpha}$-III and AA determination was prepared as follows. After an overnight fast, blood from HFH subjects (n=16) and healthy, normolipemic volunteers (n=16) was collected, and LDL was prepared by sequential density gradient ultracentrifugation by use of a previously described method that minimizes oxidation.[21] Antioxidants were not included. LDL protein concentration was determined by the Lowry method.[22] LDL cholesterol levels were

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TABLE 3. Urinary Excretion of F_2 Isoprostanes in HFH Patients and Controls

Variable	HFH	Control	*P*
$iPF_{2\alpha}$-III*	85±5.5	58±4.2	<0.0005 (n=38)
$iPF_{2\alpha}$-VI*	281±22	175±13	<0.0001 (n=38)
$iPF_{2\alpha}$-VI†	487±71	248±56	<0.05 (n=12)
AA‡	2.04±0.69	2.5±0.66	NS (n=12)

*In pmol/mmol creatinine; †pmol/nmol arachidonate; ‡nmol/mmol creatinine. Values are expressed as mean±SEM.

measured by a colorimetric assay that is certified by the Centers for Disease Control and Prevention (Sigma Chemical Co).

Statistical Analysis

Data are expressed as mean±SEM. Data from HFH and HC groups were compared with their respective controls by initial ANOVA and by 2-tailed, unpaired *t* tests for continuous variables and the Fisher exact test for categorical data, as appropriate. Grouped data were initially analyzed for covariance of slopes before calculation of pooled correlation coefficients if appropriate.

Results

Pertinent clinical characteristics and lipid profiles of patients with both HFH and HC are described in Tables 1 and 2.

Urinary $iPF_{2\alpha}$-III (pmol/mmol creatinine) was elevated in patients with HFH (85±5.5; $P<0.0005$) compared with age- and sex-matched normocholesterolemic controls (58±4.2) (Table 3). Serum cholesterol levels were 548±30 and 180±8.0 mg/dL in the HFH and control groups, respectively ($P<0.0001$). There was a significant correlation ($r=0.41$; $P<0.02$) between urinary $iPF_{2\alpha}$-III excretion and serum cholesterol for HFH patients but not for control subjects (Figure 2A). Urinary levels (pmol/mmol creatinine) of $iPF_{2\alpha}$-VI were also elevated in HFH patients (281±22; $P<0.0001$) compared with controls (175±13) (Table 3). Similarly, urinary excretion of $iPF_{2\alpha}$-VI correlated ($r=0.39$; $P<0.03$) with serum cholesterol levels in the HFH patients but not among controls (Figure 2B). There was no difference (P=NS) in urinary AA (nmol/mmol creatinine) between HFH (2.04±0.69; n=12) and control (2.5±0.66; n=12) subjects. Adjustment of $iPF_{2\alpha}$-VI excretion (487±71 versus 248±56 pmol/nmol arachidonate; $P<0.05$) for AA levels in urine maintained the distinction previously observed between the groups.

In addition to the changes observed in urinary F_2 isoprostanes, the levels (mean±SEM ng/mg protein) of $iPF_{2\alpha}$-III esterified in the LDL sampled from HFH subjects (n=16) were significantly higher (1.09±0.08) than in control subjects (0.40±0.03; n=16; $P<0.0001$) (Table 4). Notably, $iPF_{2\alpha}$-III levels in LDL and urine were correlated in HFH patients ($r=0.42$; $P<0.03$) and among controls ($r=0.44$; $P<0.01$) (Figure 3). Furthermore, $iPF_{2\alpha}$-III levels were elevated in HFH subjects (n=7) compared with controls (n=7) when corrected for LDL cholesterol (0.68±0.17 versus 0.25±0.04 ng/mg cholesterol; $P<0.05$) or LDL AA (0.32±0.08 versus 0.09±0.02 ng/mg arachidonate; $P<0.01$) (Table 4). Total (free and phospholipid-bound) LDL levels (ng/mg protein) of AA were similar (P=NS) in HFH patients (4.36±0.51) and in control subjects (4.76±0.53) (Table 4).

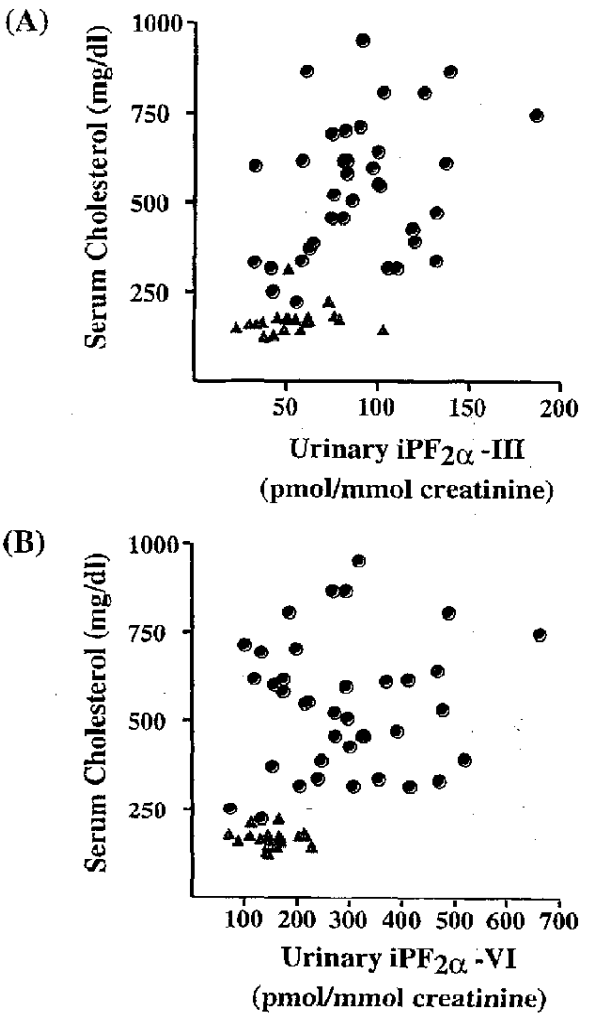

Figure 2. Correlation of fasting serum cholesterol and urinary (A) $iPF_{2\alpha}$-III ($r=0.41$; $P<0.02$) and (B) $iPF_{2\alpha}$-VI ($r=0.39$; $P<0.03$) was only significant in HFH patients (circles). These relationships were not significant among controls (triangles).

There was no significant relationship between urinary F_2 isoprostane excretion and age, sex, or a history of the use of lipid-lowering agents in HFH patients. Similarly, a history of aspirin use (n=28/38) was not associated with lower levels of urinary $iPF_{2\alpha}$-III (86±6.4 versus 79±11; P=NS) or $iPF_{2\alpha}$-VI (279±27 versus 285±38; P=NS). There were no significant differences in urinary levels of

TABLE 4. LDL Levels of $iPF_{2\alpha}$ in HFH Patients and Controls

Variable	HFH	Control	*P*
$iPF_{2\alpha}$-III			
ng/mg protein	1.09±0.08	0.4±0.03	<0.0001 (n=16)
ng/mg cholesterol	0.68±0.17	0.25±0.04	<0.05 (n=7)
ng/μg arachidonate	0.32±0.08	0.09±0.02	<0.01 (n=7)
AA, μg/mg protein	4.36±0.51	4.76±0.53	NS (n=7)

Values are expressed as mean±SEM.

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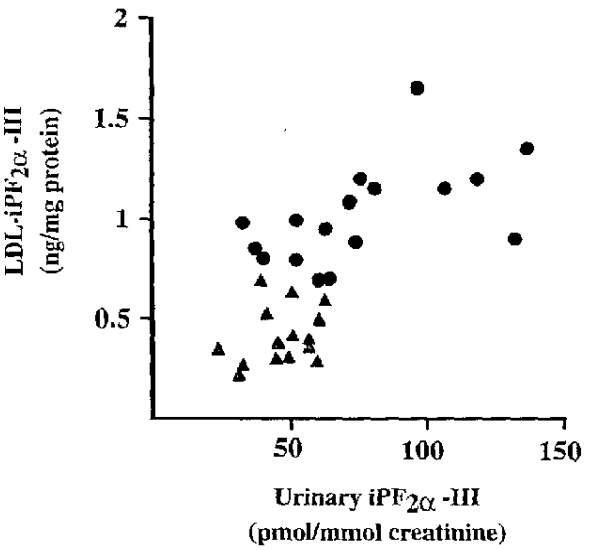

Figure 3. Urinary excretion of $iPF_{2\alpha}$-III is correlated with LDL $iPF_{2\alpha}$-III levels in HFH patients (r=0.42; P<0.03; circles) and in control subjects (r=0.44; P<0.01; triangles).

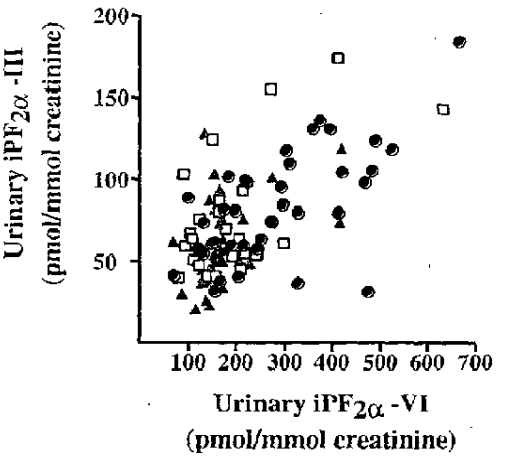

Figure 4. Correlation (r=0.57; P<0.0001; n=106) of urinary $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI levels in HFH patients (circles), HC patients (squares), and control subjects (triangles). ANCOVA failed to discriminate between the slopes describing this relationship in the individual groups.

either $iPF_{2\alpha}$-III (95±19.3 versus 77±9.2) or $iPF_{2\alpha}$-VI (295±32 versus 283±38) in the 6 patients who gave a history of antioxidant use in HFH. Neither serum cholesterol nor urinary isoprostane levels were significantly different between HFH patients with and without angiographic evidence of coronary disease.

Urinary levels of $iPF_{2\alpha}$-III (81±10 versus 59±4; P<0.05) and $iPF_{2\alpha}$-VI (195±18 versus 149±20; P<0.05) were also elevated in patients with moderate hypercholesterolemia compared with age- and sex-matched controls (Table 5). Serum cholesterol levels correlated with urinary $iPF_{2\alpha}$-III (r=0.42; P<0.03) in HC patients but not in controls.

ANCOVA failed to identify a significant difference in the slopes relating urinary levels of the 2 isoprostanes among the groups. Therefore, correlation between these variables is reported for patients and controls. Urinary excretion of $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI was significantly correlated (0.57; P<0.0001) for all hypercholesterolemic patients and control subjects (Figure 4). A similar relationship was seen within individual study groups for HFH (r=0.62; P<0.0001; n=38), HC (r=0.59; P<0.005; n=24), and control subjects (r=0.53; P<0.0005; n=44).

Discussion

Isoeicosanoids are formed by direct free radical attack on AA, a natural constituent of the phospholipid domain of cell membranes. Unlike the conventional enzymatic products of this fatty acid (the eicosanoids), these isomers are formed initially in situ in cell membranes, from which they are cleaved by phospholipases.[18] These properties have raised the possibility of their use as markers of free radical generation that can be measured both at the cellular site of oxidant stress and in biological fluids, such as plasma and urine.[23,24]

In the present study, we applied specific assays, using mass spectrometry, for 2 structurally distinct isomers of F_2 isoprostanes, $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI. We favored urine as a target matrix over plasma; it may be obtained noninvasively, and given the minor amount of AA substrate present in urine compared with plasma, it is likely to be less susceptible to isoprostane formation ex vivo. Our prior experience using both plasma and urine to estimate eicosanoid biosynthesis suggests that qualitatively distinct information is unlikely to be provided by a plasma-based assay.[25,26]

Initially, we focused on $iPF_{2\alpha}$-III. This compound is a vasoconstrictor and a mitogen and may modulate platelet function in vitro.[27,28] However, the relevance of these properties to its role in vivo remains unclear. Urinary $iPF_{2\alpha}$-III is elevated in several syndromes putatively associated with oxidant stress, including cigarette smoking[29] and vascular reperfusion.[17,30] Controlled evaluation indicates that it is suppressed by antioxidant vitamins.[29,31,32] The concentration of $iPF_{2\alpha}$-III is elevated in human atherectomy specimens compared with levels in normal arterial segments, and we have immunolocalized the compound to monocyte/macrophages and vascular smooth muscle cells in such tissue.[14] Given these observations, we wished to address the hypothesis that formation of $iPF_{2\alpha}$-III, as reflected by its urinary excretion, might be elevated in hyperlipidemia.

Urinary levels were increased in young patients with HFH compared with age- and sex-matched controls. There was no difference in urinary arachidonate between the 2 groups, which indicates that this was not merely a differ-

TABLE 5. Urinary Excretion of F_2 Isoprostanes in HC Patients and Controls

Variable	HC	Control	P
$iPF_{2\alpha}$-III	81±10	59±4	<0.05 (n=24)
$iPF_{2\alpha}$-VI	195±18	149±20	<0.05 (n=24)

Values are expressed as pmol/mmol creatinine (mean±SEM).

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ence in substrate availability. We have also demonstrated that F_2 isoprostanes are not generated by auto-oxidation of lipid ex vivo when urine is stored under the conditions described in the present study.[19] Participants in the present study were not placed on controlled diets. Thus, the precise role of dietary lipids in isoprostane generation remains to be addressed definitively. The magnitude of the increment in $iPF_{2\alpha}$-III excretion over control values in these patients, all of whom were nonsmokers, was similar to that observed in apparently healthy individuals who were moderate (>15 cigarettes/d) cigarette smokers.[29] Given the potentially distinct mechanisms that might result in free radical generation in smokers,[33] one might anticipate an even greater increment in dyslipidemic individuals who smoke cigarettes.

We extended the study to address this hypothesis in a sample of patients with a more common form of dyslipidemia. Excretion of $iPF_{2\alpha}$-III was also elevated, although to a lesser extent, in HC patients compared with a control group. It is noteworthy that the HFH and HC groups in these studies were distinct from each other, not only in terms of cholesterol levels but also by virtue of age, gender distribution, cigarette smoking, and presence of coronary disease. Thus, the relatively small differences in urinary F_2 isoprostanes between the groups, despite major differences in serum cholesterol levels, may reflect the confounding influence of variables such as age, diet, or smoking. Although a history of supplementary intake of a variety of antioxidant drugs and doses did not detectably influence the levels of urinary F_2 isoprostanes, such a history was not assessed in a controlled manner in the present study. By contrast, we have previously shown that antioxidants do suppress elevated isoprostanes both in vitro and in vivo.[19,29,31,32,34] The quantitative relationship between isoprostane generation and variables such as the extent of coronary disease, age, sex, ethnicity, nutritional status, and drug consumption in hypercholesterolemic patients requires larger studies designed and controlled specifically to address these issues. Interestingly, despite the lack of control for diet and medications and the small sample sizes, urinary excretion of $iPF_{2\alpha}$-III exhibited a weak but statistically significant correlation with fasting serum cholesterol in both groups of hypercholesterolemic patients, albeit not in controls, further facilitating its applicability to the detection of oxidant stress in large samples.

In the present study, $iPF_{2\alpha}$-III, esterified in LDL, was elevated in a subset of the homozygotes compared with matched controls. This was true whether $iPF_{2\alpha}$-III levels were corrected for LDL protein or cholesterol, which suggests that elevated levels are not dependent on differences in LDL cholesterol. More importantly, HFH LDL $iPF_{2\alpha}$-III levels remained elevated compared with controls when corrected for LDL AA. Although it is unknown if circulating LDL might represent a source of augmented urinary F_2 isoprostanes in such patients, the levels of $iPF_{2\alpha}$-III in LDL and urine were significantly correlated in the HFH patients.

Despite evidence consistent with the value of urinary $iPF_{2\alpha}$-III as an index of oxidant stress, a potential caveat is its capacity for formation as a minor product of both cyclooxygenase (COX) enzymes.[20,34] We have previously shown that COX-1 in platelets exhibits the potential to form $iPF_{2\alpha}$-III but not other F_2 isoprostanes in this manner.[20] Similarly, monocyte COX-2 may be a source of the compound.[34] It seems likely that these pathways contribute trivially to actual generation of $iPF_{2\alpha}$-III in vivo, as reflected by its excretion in urine. Thus, we have reported that treatment of cigarette smokers with aspirin, a nonspecific COX inhibitor,[35] will depress excretion of thromboxane metabolites but not of $iPF_{2\alpha}$-III in urine.[29] Aspirin and indomethacin both fail to depress urinary $iPF_{2\alpha}$-III in healthy volunteers.[19]

Despite these observations, it seemed prudent to develop methods to measure another F_2 isoprostane that was not susceptible to enzymatic formation. $iPF_{2\alpha}$-VI is a member of a distinct class of F_2 isoprostanes.[36,37] It is not formed by COX, and its excretion in volunteers is not suppressed by aspirin. Urinary levels of $iPF_{2\alpha}$-VI are higher than those of $iPF_{2\alpha}$-III.[19]

Urinary $iPF_{2\alpha}$-VI was also increased in both groups of dyslipidemic patients. Urinary levels of the 2 F_2 isoprostanes were highly correlated. This observation is consistent with the hypothesis that excretion of both compounds in urine reflects formation by a common mechanism: free radical–catalyzed generation of prostaglandin isomers. Recently, we have observed a similar relationship in urinary levels of patients undergoing coronary reperfusion.[30] This correlation and the absence of any relationship between aspirin use and urinary levels of both F_2 isoprostanes in HFH patients argue against COX-dependent formation of $iPF_{2\alpha}$-III in vivo in HFH. Given that immunoassays are currently available only for $iPF_{2\alpha}$-III,[38] this observation is relevant to clinical investigation. This is especially so because COX activation may coincide with oxidant stress in clinical settings such as ischemia-reperfusion syndromes[39] and in cigarette smoking.[40] Recently, Davi et al[41] reported that urinary levels of immunoreactive $iPF_{2\alpha}$-III are elevated in hypercholesterolemia. Given that chemically synthesized standards for most of the 64 F_2 isoprostanes and none of their potential metabolites have been checked for cross-reactivity with this material, our results not only extend these observations but provide important support for their findings. We demonstrate that authentic $iPF_{2\alpha}$-III is elevated in both moderate and severe hypercholesterolemia. Furthermore, our data indicate that this does not reflect auto-oxidation of increased amounts of substrate ex vivo in the urine of hypercholesterolemic patients but rather its increased generation in vivo. Finally, the concomitant increase in urinary $iPF_{2\alpha}$-VI is consistent with formation of $iPF_{2\alpha}$-III by a free radical–dependent pathway rather than as a product of COX turnover.

In summary, we report that urinary excretion of 2 F_2 isoprostanes is elevated in patients with hypercholesterolemia. Attempts to establish the efficacy of aspirin through clinical trials in cardiovascular disease were frustrated for more than a decade because populations were diluted with respect to likely benefit from the drug.[42] The development

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of methods to estimate thromboxane biosynthesis using urinary metabolites not only aided in the selection of low doses of aspirin for cardiovascular indications[42] but also identified unstable angina[43] and therapeutic thrombolysis[44] as biochemically rational targets for aspirin therapy, a contention borne out by the results of randomized clinical trials.[45,46] Clinical trials of antioxidants have been performed in the past without a clear in vivo biochemical rationale, either for the choice of clinical targets or for the selection of antioxidant doses of such compounds. Given their formation in LDL oxidized in vitro, their presence in human atherosclerotic plaque, and now their augmented formation in circulating LDL and in urine of patients with dyslipidemias, it is likely that measurement of F_2 isoprostanes may prove useful in the exploration of the importance of oxidant stress and its modulation in human atherosclerotic disease.

Acknowledgments

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